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(71) Applicant *(for all designated States except US)*: MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : WISCHIK, Claude, Michel [AU/GB]; 6 Herbert Street, Cambridge CB4 1AQ (GB). MILSTEIN, Cesar [GB/GB]; 292A Hills Road, Cambridge CB2 2QG (GB). KLUG, Aaron [GB/GB]; 70 Cavendish Avenue, Cambridge CB1 4UT (GB).

(74) Agent: HALLYBONE, Huw, George; Carpmaels & Ransford, 43 Bloomsbury Square, London WC1A 2RA (GB).

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(54) Title: PAIRED HELICAL FILAMENT CORE AND ANTIBODY THERETO USEFUL IN DIAGNOSING ALZHEIMER'S DISEASE

(57) Abstract

Brain tissues of patients suffering from Alzheimer's disease show characteristic lesions, including those called neurofibrillary tangles. The tangles comprise dense accumulations of paired helical filaments (PHFs). This invention provides a pronase-resistant PHF core which remains after removal of peripheral PHF constituents by pronase. Unlike whole PHFs, the PHF core provides an antigen uniquely associated with neurofibrillary tangles. The invention includes antibodies to the PHF core useful in immunoassays for the core and further includes the diagnosis of Alzheimer's disease using such an immunoassay.

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PAIRED HELICAL FILAMENT CORE AND ANTIBODY THERETO USEFUL IN DIAGNOSING ALZHEIMER'S DISEASE

This invention relates to a protein associated with senile dementia of the Alzheimer type, precursors of the protein and an antibody thereto. The antibody may be used in a method of diagnosis of Alzheimer's disease.

Senile dementia of the Alzheimer type (Alzheimer's disease) is an age-related disorder affecting approximately 4% of those aged over 65 in the UK. Alzheimer's disease causes progressive cognitive impairment and invariably results in death. Post mortem studies of brain tissue show various characteristic lesions discovered by Alzheimer in 1906. The main lesions which occur in the brain are neurofibrillary tangles (tangles) neuritic plaques (plaques) and granulovacuolar degeneration (for a review see: Tomlinson B.E. "The ageing brain" in Recent Advances in Neuropathology, No. 1, eds. Smith W.T. and Cavanagh J.B. Churchill Livingstone, London, 129-159). It has been shown that tangles comprise dense accumulations of an ultrastructurally distinct entity which has been named a paired helical filament (PHF) (Kidd M. (1964), Brain 87, 307-320; Wisniewski H.M. et al (1976), J. Neurol. Sci., 27, 173-181 and (1979), Ann. Neurol., 5, 288-294). The paired helical filaments have been shown to consist of a double helical stack of transversely-oriented subunits, giving the overall shape of a ribbon twisted into a left-handed helix (Crowther R.A. et al (1985) EMBO J. 4(13B), 3661-3665; Wischik C.M. et al (1985), J. Cell Biol. 100, 1905-1912; Wischik C.M. et al (1986), Brit. Med. Bull 42(1), 51-56).

It has been shown that antibodies raised against neurofilaments are capable of cross-reacting with paired helical filaments isolated from tangles (Ihara, Y. et al (1981), Proc. Jap. Acad. 57, 152-156; Anderton B.H. et al (1982), Nature 298, 84-86; Gambetti P.G. et al (1983), J. Neuropathol. Exp. Neurol. 42, 69-79). More recent data

has suggested however that neurofilament reactivity of the PHFs is, in fact, due to cross-reactivity between neurofilament and tau-derived epitopes (Luca F.C. et al (1986), PNAS 83, 1006-1010; Sternberger N.H. et al (1985), PNAS 82, 4274-4276). In addition, several groups have reported the extraction of tau protein from partially purified tangel fractions (Kosik K.S. et al (1986) PNAS 83, 4044-4048; Gorevic P.D. et al (1986), J. Neuropath. Exp. Neurol. 45, 647-664; Grundke-Iqbal I. et al (1986), J. Biol. Chem. 261, 6084-6089). There is some dispute as to whether neurofilament reactivity survives harsher extraction of PHFs (Rasool C.G. et al (1984), Brain Res. 310, 249-260) although Tau reactivity clearly survives SDS extraction (Bignami A. (1984), Acta, Neuropathol. 64 243-250).

There is no available laboratory diagnostic test for Alzheimer's disease in the living body. Recently, the existence of antigenic paired helical filament protein in the cerebrospinal fluid (CSF) was reported (Mehta P.D. et al, The Lancet, 6th July 1986) and it was shown that using an enzyme immunoassay including a so-called anti-PHF antibody, it was possible to measure a higher level of antigen in the CSF of Alzheimer's disease patients than in the CSF of other neurological patients. The assay was not however definitive, there being considerable overlap of the spread of results. More importantly, it has now become clear that the antibody is in fact directed against ubiquitin and will cross-react with ubiquitin in the CSF.

We have now discovered that isolated paired helical filaments consist of two structurally distinct components: a pronase resistant core which wholly contains the structural subunit of the PHF and accounts for some 100 Kd per subunits, and protease sensitive material which appears in electron micrographs as a poorly defined

("fuzzy") peripheral region of the PHF. The fuzzy outer coat of the PHF accounts for approximately 20 Kd per subunit, and is immunodominant in the sense that the fuzzy coat occludes epitopes intrinsic to the core of the PHF. Antibodies which have been claimed to react with PHFs and which cross-react with other proteins such as the microtubule associated protein tau, and perhaps H and M neurofilament proteins and ubiquitin, are directed against epitopes which are present only in the fuzzy coat of the PHF. We have shown that the pronase resistant core (hereinafter referred to as "the PHF core protein") is not susceptible to cross-reaction with such antibodies. The term "PHF core protein" is not intended to imply that the core consists of a single protein, but rather is to be understood as indicating the proteinaceous core of PHF without specifying the structure of the core.

According to the present invention, there is provided a paired helical filament core protein substantially free of other proteinaceous material.

Pronase is a broad-spectrum protease derived from Streptomyces griseus and comprises a mixture of enzymes. Pronase has a specific activity of 70,000 proteolytic units per g dry weight. (One unit is defined as the amount of enzyme that liberates a digestion product equivalent to 25 ug of tyrosine per minute at 40^{o}C, pH 7.5).

The protein of the invention comprises the proteinaceous PHF core, without the attached immunodominant protein layer. This core protein has been shown to comprise a number of different fragments which is believed to comprise a plurality of subunits arranged transversely to form a double helical polymer twisted into a left-handed ribbon. Each subunit appears to consist of three domains arranged in a "C" shape.

In a third aspect of the invention, there is provided a precursor or fraction of the paired helical filament core protein, substantially free of other proteinaceous material. The subunit is a precursor of the paired helical filament core protein. Such a fraction has been located as a soluble protein from normal brain tissues and also from the cerebrospinal fluid of Alzheimer patients. The further purification of the pronase resistant core to separate core precursors or fractions is described hereinafter.

A monoclonal antibody raised against the PHF core protein or PHF core protein precursor (such antibodies being included in the invention) fails to label cytoskeletal structures seen in histological sections and anticytoskeletal antibodies which label isolated PHFs fail to label the PHF core that has been stripped of the fuzzy outer domain which occludes core epitopes in situ. These findings suggest that published antibody selection strategies based on histological tangle reactivity have failed to identify protein(s) which make up the PHF core, and which, in the light of the subunit structure of the PHF, is likely to be responsible for the property of aberrant assembly. Also the known antibodies to PHF are susceptible to cross-reaction with antigens normally found in CSF, rendering assays employing them at best difficult to interpret and in any event open to false positive results. Thus the protein of the invention provides an antigen uniquely associated with neurofibrillary tangles which can be used to assay for PHF, by raising antibody to the protein or a precursor thereof. Antibody may also be raised against the anti-PHF core antibody or anti precursor antibody. The assay may be conducted by labelling the antibody and detecting formation of the antibody/antigen complex, or a competitive technique may

be used. For example, the labelled antibody may be either the antibody (preferably monoclonal antibody) having specificity for the PHF core protein or PHF core protein precursor, or it may be an antibody having specificity for the antibody having specificity for the core protein or precursor. The assay may also be conducted using labelled antigen to detect an antibody.

According to a fourth aspect of the invention, there is provided an antibody to the protein of the first or second aspect of the invention.

The antibody may be a polyclonal antibody, but is preferably a monoclonal antibody. The monoclonal antibody specifically described herein is designated by its clone number, 423.

In a further aspect of the invention there is provided a process for the preparation of a protein of the first aspect of the invention, comprising the steps of

i) isolating paired helical filaments from Alzheimer neurofibrillary tangles and

ii) digesting the isolated paired helical filaments with pronase and

iii) purification of the PHF core protein.

Figure 1 shows the nucleotide and predicted amino acid sequences of a PHF protein deduced using cDNA probes prepared by a method involving a synthetic nucleotide probe derived from an amino acid sequence of part of a PHF core protein, and

Figure 2 shows the amino acid sequence of a peptide fragment of the PHF core.

The invention includes PHF core and core fractions irrespective of the method of preparation and therefore includes PHF core and core fractions preparable using the method of the invention. Accordingly, the invention

encompasses materials sharing essential characteristics of the products prepared using our method. A yet further aspect of the invention is material having immunological characteristics and, as appropriate, eletron microscopic structural characteristics corresponding to the core and core fractions prepared using the method of the invention.

The invention further provides a method for the diagnosis of Alzheimer's disease comprising assaying a sample, for example cerebrospinal fluid (CSF), for the presence of the paired helical filament core protein or of a precursor or fraction thereof.

The PHF core protein of the invention provides an antigen uniquely associated with neurofibrillary tangles and may therefore be used to diagnose Alzheimer's disease. The core protein, i.e. that part of the structure remaining after Pronase treatment, may be isolated by the procedure described below by way of example only.

Tissue containing neurofibrillary tangles is dissected from the brains of patients who have died from Alzheimer's diseases, as confirmed by necropsy. The tissue is homogenised and filtered. The filtered homogenate is subjected to a series of (e.g. three) gradient centrifugation steps, the centrifugation preferably being sucrose gradient centrifugation. There is obtained from the centrifugation a fraction (hereinafter denoted "ifI") enriched in tangles and tangle fragments, that is the centrifugation isolates the paired helical fragments. Stages in the preparation of PHFs may be monitored by counts of whole tangles and tangle fragments, using the distinctive gross morphology of tangles observed by fluorescence microscopy (Yen, S-H.C.,F. Gaskin and R.D. Terry. 1981. Immunocytochemical studies of neurofibrillary tangles. Am. J. Pathol. 104:77-89).

The tangles and tangle fragments of fraction ifI are digested with pronase and optionally with nuclease, e.g. micrococcal nuclease, to remove further contaminants. Finally, the tangle fragments are subjected to further centrifugation to further separate them from pronase- and nuclease-resistant fragments. The skilled reader will immediately understand that alternative proteases to pronase may be used, in that "pronase" is generally understood to be a generic term for proteases.

The tangle fragments after digestion with pronase and optionally nuclease are subjected to gradient centrifugation to purify them. For good results, the gradient, in practice a linear sucrose gradient, ranges from 1.18 to 1.05, which optimises the velocity separation of tangle fragments while floating off unwanted contaminants at higher levels of the tube. Typically, the sucrose gradient is poured over a CsCl cushion and the enriched fraction is collected at the CsCl interface at density 1.45.

The PHF core component may be further purified to separate core fractions by, for example, contacting the ifII with formic acid or subjecting it to succinylation. The use of sonication further improves the purification of the PHF core protein. The end product of the process is a soluble residue of paired helical filaments. The residue may be separated by gel electrophoresis into a number of PHF core precursors. The only fragment bands seen by Coomassie stain or silver stain correspond to bands which are western blot positive with antibody 423.

The isolated PHF core protein may be used according to the procedure of Kohler and Milstein (Nature 298 (1975)) to prepare a monoclonal antibody to the PHF core component. The antibody we selected was designated by its clone number, 423, but the specific monoclonal

antibody used is not critical to the invention. What is important is that the procedure may be repeated to obtain a monoclonal antibody to the PHF core portion.

Antibody 423 does not label cytoskeletal structures and anticytoskeletal antibodies which label isolated PHFs do not label the PHF core. Antibodies to the PHF core component therefore appear to be useful for providing an assay sensitive only to neurofibrillary tangles and, hence, suitable for diagnosing Alzheimer's disease.

The assay for the PHF core protein may be conducted in accordance with any known immunoassay technique, for example. The immunoassay may be homogeneous or heterogeneous. Suitable labels for detecting the presence of antibody/antigen complex include radiolabels, fluorescent labels and enzyme labels. The assay may, for example, be a radioimmunoassay or ELISA. A less preferred alternative to using a monoclonal antibody is to use a polyclonal antibody.

The present invention may be used to prepare probes to nucleotide sequences corresponding to PHF core protein or a core protein precursor. For example, a cDNA library or portion thereof, prepared from the frontal cortex of a patient, may be screened by using an oligo- or poly-nucleotide probe corresponding to part of the polynucleotide sequence encoding a PHF core protein or peptide. The probe may be labelled with $[\gamma-^{32}P]$ ATP by using T4 polynucleotide kinase. Hybridization-positive clones may be plaque-purified and, if desired, sequenced. It is contemplated that the cDNA clone or a fragment thereof may itself be used to diagnose Alzheimer's disease as well as for research purposes.

The invention therefore includes nucleotide probes corresponding to at least part of the PHF core or a core fraction. The probe may be of synthetic or cDNA origin. Preferably, the cDNA library is screened using a synthetic

nucleotide probe derived from an amino acid sequence of part of a PHF core protein or peptide. The probe will be of sufficient length to hybridize and generally comprises fifteen or more adjacent nucleotides from the sequence to which the probe corresponds, for example any sequence of fifteen nucleotides corresponding to part of the peptide sequence shown in Figure 2.

Recently published work relating to the use of nucleotide probes in the characterisation of components of the PHF core has disclosed that a portion of the core is formed by part of tau or a tau-like protein. However, monoclonal antibody 423 does not react with intact isolated mammalian tau protein, and reacts weakly with untreated PHFs. Thus monoclonal antibody 423 recognises the tau sub-sequence found in the core protein, which subsequence had not hitherto been isolated. (M. Goedert et al. (1988), PNAS 85, 4051-4055; C.M. Wischik et al. (1988), PNAS 85, 4506-4510; C.M. Wischik et al (1988), PNAS 85, 4884-4888; see also BMJ 297, p.444 (1988)).

All references referred to herein are incorporated herein by reference.

EXAMPLE

PREPARATION OF THE PHF CORE PROTEIN AND MONOCLONAL ANTIBODY THERETO

Brains were obtained post mortem from well-documented patients with a clinical diagnosis of senile dementia of the Alzheimer type. In each case, the clinical diagnosis was confirmed histologically by the presence of large numbers of plaques and tangles in frontal and temporal cortex. Tissues obtained from four human brains were used in the present study; these people had died at 65, 67, 71 and 84 years old. The material used in preparations was taken from frontal cortex,

temporal cortex, and hippocampus. Transverse sections were cut out after removal of pial and meningeal membranes. White matter was then dissected away and discarded, leaving 20-40 gm of tissue for use in an individual preparation. Tissues were stored at -70°C. Tissues were mixed with an approximately equal volume of 0.32 M sucrose, 1mM magnesium chloride, 0.25 mM phenylmethyl sulfonyl fluoride (PMSF), 1mM EGTA and 5mM potassium phospate (pH 6.5). The resulting mixture washomogenised in a PTFE-glass manual homogeniserand filtered through a four-fold muslin screen mounted on a syringe, to give a final volume of 70-100 ml. The filtered homogenate was layered over an equal volume of 1.5 M sucrose, 1mM magnesium chloride, 0.25 mM PMSF, 1mM EGTA, 5mM potassium phosphate (pH 6.5), and centrifuged at 27,000 rpm for 60 min in a Beckman SW27 rotor (Beckman Instruments, Inc., Palo Alto, California) at 15°C. The supernatant and pellet were discarded. The material at the interface was harvested, together with the underlying 1.5 M sucrose layer. This mixture was rehomogenised in a PTFE-glass homogeniser, layered over 2.0 M sucrose, 1mM magnesium chloride, 0.25 mM PMSF, 1 mM EGTA, 5mM potassium phosphate (pH 6.5) and centrifuged at 40,000 rpm for 60 min in a Beckman SW40 rotor at 15°C. The fraction that floated to the top and the upper supernatant were discarded, as was the bottom pellet. The material at the interface was harvested, resuspended in the initial 0.32 M sucrose and buffer solution, and centrifuged at 40,000 rpm for 60 min in a Beckman SW40 rotor at 15°C. This final pellet known as ifI material, was stored at -70°C, using the cut polyallomer centrifuge tube sealed with plastic film as a storage well.

The further procedure for obtaining a fraction known as ifII that is highly enriched for pronase treated PHFs, is as follows. Each ifI pellet is homogenised in a 9 ml

volume of buffer consisting of 20 mM Tris pH 7, 1mM $CaCl_2$ and 3mM $MgCl_2$. To this, 100 ul of micrococcal nuclease (200 units per ul) in 20 mM Tris pH 7 and 1 mM $CaCl_2$ is added and the mixture is digested for 1 hr at 35°C. After 1 hr, 100 ul of pronase made up as 10 mg per ml in 20 mM Tris pH 7, 1mM $CaCl_2$ and 3 mM $MgCl_2$, and digested at 35°C for a further hour. Digestion is stopped by adding 150 ul of 200 mM EGTA and 150 ul of 200 mM EDTA. To this mixture is added 0.5 ml of 2M NaCl making a final concentration of100 mM, 100 ul of 3 M Tris pH 8.8 and 100 mg cholic acid. Following vigorous mixing, the mixture is filtered through a Bellatini 8 glass bead filter made upin a Pasteur pipette. Next, 0.7 g sucrose is added to the mixture to bring its density to 1.18. A second solution is prepared containing 14% sucrose (density 1.05) in 20 mM Tris pH 7, 2mM EGTA, 2mM EDTA. A third solution, consisting of 68% CsCl at a density of 1.45 in the same buffer (20 mM Tris pH 7, 2 mM EGTA, 2 mM EDTA) is prepared. A gradient is poured in a 13 ml SW40 Ultraclear centrifuge tube over a 2 ml cushion of the CsCl solution. The gradient ranges from 1.18 up to 1.05. This particular gradient is very important since it optimises the velocity separation of tangle fragments collecting at the CsCl interface while floating off unwanted contaminants at higher levels of the tube. The tube is spun at 40,000 rpm for 3 hours in a Beckman SW40 rotor at 15°C. The pronase-treated PHF-rich layer known as ifII forms at the CsCl interface at density 1.45 and is carefully collected by inserting a syringe needle just below the interface. All the ifIIs are collected in SW28 centrifuge tubes (3 ifIIs per tube) and diluted tenfold in 5 mM citric acid, 5 mM Na_2HPO_4 at pH 5.5. These are spun in an SW28 rotor at 28,000 rpm at 15°C overnight. The supernatants are discarded and pellets are collected for further biochemical extraction.

PHF core protein may be further purified by sonication in the presence of a suitable reagent, e.g. formic acid or a succinylating agent. An exemplary procedure is as follows. The pellets from the SW28 spin are collected with residual citrate phosphate buffer (18 ifIIs per 2 ml) and sonicated extensively using an E/MC Corp. Microprobe Sonicator at full power, tuning 3-4. This material is transferred to a 5 ml SW50 polyallomer tube, the volume being brought up to 5 ml with water, and following further sonication, the material is centrifuged in a Beckman SW50 rotor at 50,000 rpm at 15^{o}C for 1 hr. The supernatant collected after this spin is known as Alz 5.5. To the pellet, 1 ml 100% formic acid is added and is sonicated extensively using the probe sonicator mentioned above. This material is transferred to a 1 ml Ependorf tube and is centrifuged for 10 min in an Ependorf benchtop centrifuge. The supernatant of this spin is harvested and is lyophilised to dryness. The pellet is discarded. To the lyophilisate, 1 ml of ammonium acetate buffer at pH 5.5 (30 ul of glacial acetic acid in 50 ml of glass distilled water brought to pH 5.5 with ammonium hydroxide) is added. This suspension is sonicated extensively using a probe sonicator and centrifugation in an Ependorf benchtop centrifuge is performed. The supernatant obtained from this step contains purified (fractionated) Alzheimer PHF core protein. This fraction is known as Alz. F.5.5.

Alz. F.5.5 was subjected to gel electrophoresis and immunoblotting. A number of fragments were separated into different bands by the electrophoresis and the only bands seen by coomassi stain or silver stain correspond to bands which are western blot positive with antibody 423. The pellet from this last spin may be recycled through a formic acid extraction, lyophilisation and re-extraction at pH 5.5.

The monoclonal antibody was prepared using the procedure of Kohler and Milstein (Nature 298 (1975)).

Seventy mice were injected over a period of 24 months. Thirty five of the animals were injected with ifII, and of the remainder roughly half were injected with ifI and half with a fraction derived from ifII. Five mice produced sera which distinctly labelled PHFs from which the fuzzy outer domain had been removed. Splenic fusions were prepared from a total of nine animals over this period. A variety of screening techniques were employed in selecting positive clones and sera, including in situ tangle reactivity, isolated tangle reactivity, decoration of isolated PHFs as seen by immuno-electron microscopy and labelling of purified PHF core protein fractions. Standard immunolabelling protocols and western blotting protocols were used. From a starting total of ten positivehybridomas, 1 clone (derived from an ifII-injected animal and hereinafter referred to as 423) with strong anti-PHF core reactivity as seen by electron microscopy survived to the stage of bulk preparation of pure antibody in serum-free medium.

MORPHOLOGY OF FILAMENTS

PHFs prepared by the method which includes a pronase digestion step (ifII) differ from filaments prepared without proteolysis (ifI) by the loss of a fuzzy outer domain seen as an area of increased deposition of negative stain along the PHF border. PHFs stripped of this fuzzy outer border by pronase nevertheless retain all the finer structural features we have reported earlier as comprising the structural subunit of the PHF. Indeed longitudinal striations merging from 3 to 4, and transverse striations at approximately 3 nm intervals are more clearly revealed in pronase-treated filaments than in untreated filaments. Thus the 3-domained subunit reported earlier survives pronase treatment, and this has been confirmed by image reconstruction based on pronase-treated filaments.

ANTIBODY TO CORE PROTEIN

Antisera, oligoclonal and monoclonal hybridomas were chosen in the first instance on the basis of ability to produce unequivocal decoration of the PHF core structure as seen by electron microscopy. Screening on the basis of histological tangle reactivity proved to be unreliable as a means of predicting clones with PHF core reactivity. Indeed, supernatants able to produce PHF core labelling failed until final purification and bulk preparation of monoclonal antibody to produce labelling of tangles in appropriately prepared histological sections.

All of the hybridomas able to produce antibodies with strong decoration of the PHF core showed a characteristic helical deposition of gold particles along the filament, suggesting a regular and repetitive helical disposition of the epitope.

Non-pronase treated (ifI) PHFs could be decorated with 423, but at lower dilution than required for decoration of ifII filaments. This implies that the PHF core epitope required for 423 reactivity is probably partially occluded by proteins present in the fuzzy domain of ifI PHFs. On the other hand, positive 423 labelling of ifI PHFs implies that the 423 epitope is revealed by pronase, but is not created by proteolysis. This interpretation is also consistent with findings at the histological level.

AMYLOID FILAMENTS

The pronase-treated PHF-enriched fraction used as immunogen contained amyloid deposits collagen and lipofuscin in addition to PHFs. Antibody from hybridomas selected for PHF core reactivity failed to decorate amyloid fibrils present in the same material. Thus the proteins present in the core of the PHF are antigenically distinct from amyloid. Antibody 423 also failed to give

any histological labelling of vascular amyloid deposits present in arterioles or in degenerating capillaries present in plaque cores.

HISTOLOGY

Following large scale preparation of antibody 423, it was possible to show that only tangles are positive in cryosections from freshly frozen Alzheimer brain tissues (frontal and temporal cortex, hippocampus) following ethanol fixation and pronase treatment of the section. However, in sections fixed with paraformaldehyde, strong staining of normal non-tangle bearing pyramidal cells and smaller neurones was found. This staining was abolished by pronase treatment, and could not be seen in ethanol fixed sections without paraformaldehyde. Staining was restricted to the cell body. Dendritic and axonal staining was negative. Staining of these cells could be observed in two juvenile controls, where staining in nerve processes was completely absent.

Staining purkinje cell bodies and cell bodies of a smaller class of neurones was seen in the cerebellum. Likewise, cell body staining in the spinal cord was restricted to nucleus proprius cells. Staining of tangle-bearing neurones in paraformaldehyde fixed sections was variable, with a complete absence of staining in some cases, and co-localisation of staining with tangles in others.

COMPARISON WITH NEUROFILAMENT AND TAU ANTIBODIES

The staining pattern observed with antibody 423 can be contrasted with the staining pattern observed with anti-neurofilament and anti-tau antibodies. In the case of neurofilament antibodies which label isolated PHFs, failure of purkinje cell body labelling and positive labelling of basket cell processes serving purkinje cells

is characteristic. Likewise, the staining pattern observed with antibody 423 is distinct from either the axonal or dendritic distribution of phosphorylated or non-phosphorylated tau respectively.

Although anti-tau and anti-neurofilament antibodies label isolated PHFs washed with SDS, reactivity to both antibodies is lost after trypsin digestion of PHFs. In the present work, ifI PHFs were weakly labelled with an anti-tau antibody. This labelling was lost after pronase treatment. Weak labelling with an anti-neurofilament monoclonal (RT97) was likewise observed against ifI PHFs. This reactivity was lost after pronase digestion.

Construction of cDNA Libraries and Screening

RNA was isolated, by a modification of the guanidinium isothiocyanate/hot phenol technique (Feramisco, J.R. et al. (1982) J. Biol. Chem. 257, 11024-11031), from the brain of a 15-week-old human fetus and from the frontal cortex of a 65-year-old patient who had died with a histologically confirmed diagnosis of Alzheimer disease; the cortical tissue was obtained 3 hr after death. Poly(A)$^+$ RNAs were enriched for by oligo(dT)-cellulose affinity chromatography. First-strand cDNA synthesis was carried out by using murine reverse transcriptase in the presence of actinomycin D (40 ug/ml) with oligo(dT) as a primer. Double-stranded cDNA was generated by a modification of the procedure described by Gubler and Hoffman (Gubler, U. and Hoffman, B.J. (1983) Gene 25, 263-269), using RNase H, DNA polymerase I, and Escherichia coli DNA ligase. After treatment with S1 nuclease and EcoRI methylase, the size-selected double-stranded cDNA was cloned into the imm^{434} EcoRI insertion vector λgt10 (25) with EcoRI linkers. Fetal poly(A)$^+$ RNA (10 ug) yielded a library of 4 x 10^6 clones and 10 ug of frontal cortical poly(A)$^+$ RNA resulted in

6.2×10^6 clones.

Replica filters were screened with two mixed synthetic oligonucleotide probes, [5' GG$\binom{T}{C}$TT$\binom{A}{G}$TA$\binom{T}{C}$AC$\left(\begin{smallmatrix}A\\G\\T\end{smallmatrix}\right)AT\binom{T}{C}$TG 3'] and [5' GG$\binom{T}{C}TT\binom{A}{G}TA\binom{A}{G}AC\left(\begin{smallmatrix}A\\G\\T\end{smallmatrix}\right)AT\binom{T}{C}$TG 3'], derived from the amino acid sequence Gln-Ile-Val-Tyr-Lys-Pro of part of a PHF core protein whose sequence determination is described below.

The probes were labelled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Hybridization-positive clones were plaque-purified and the melting profile of the hybrids was determined by using 3 M tetramethylammonium chloride/50 mM Tris.HCl, pH 8.0/2 mM EDTA, containing 1 mg of $NaDodSO_4$ per ml.

A single hybridization-positive clone was obtained from 650,000 clones; the melting temperature of the hybrids was 56°C, suggesting a perfect match of the cDNA clone with one of the oligonucleotide probes. This was confirmed by sequencing a 160-base pair hybridization-positive Hae III/Alu I fragment of this clone. It was found to encode the peptide sequence, only part of which had been used for designing the oligonucleotide probes. Screening of approximately 50,000 clones from the fetal brain cDNA library with the Hae III/Alu I fragment resulted in 34 additional positives, several of which were plaque-purified. Two of these clones (λPHF5 and λPHF7) were further characterized. The exact insert size of λPHF5 is unknown, as it could not be excised with EcoRI. However, a partial restriction map indicates a length of 2.9 kb. The cDNA insert of λPHF7 is 2.8 kb long.

Nucleotide Sequence of cDNA Clones
and Deduced Amino Acid Sequence

A partial nucleotide sequence determined from clones PHF5 and PHF7, which encompasses the complete coding

region, is depicted in Fig.1. It consists of 37 nucleotides of 5' untranslated region, an open reading frame of 1056 nucleotides, an in-frame stop codon, and 12 nucleotides of 3'untranslated sequence. The translation initiation site was assigned to the methionine codon numbered as nucleotides 1-3, as it is the first ATG downstream of an in-frame stop codon (nucleotides -9 to -7). The open reading frame encodes a protein of 352 amino acids. The protein sequence is highly homologous to the sequence of mouse tau and thus constitutes the human equivalent of mouse tau.

Protein Sequence Analysis

As stated above, Alz. F.5.5 was subjected to gel electrophoresis and immunoblotting. Two major bands of 9.5 and 12 kDa were identified by antibody 423. For sequence analysis, the 9.5 and 12-kDa bands were transferred to polyvinylidene difluoride membrane and stained with Coomassie blue. Then they were excised, eluted, and digested with trypsin, and the resulting peptides were fractionated by microbore HPLC. The sequences of tryptic peptides from the 9.5- and 12-kDa fragments were determined with an Applied Biosystems gas-phase sequencer with "on-line" HPLC detection of phenylthiohydantoin=derivatized amino acids. The sensitivity of this instrument had been increased by a modification to permit 90% of the phenylthiohydantoin-derivatized amino acid released at each cycle to be analyzed. Amino acid sequences (Fig.2) derived from both the 9.5- and 12-kDa components were found to overlap with each other.

The protein sequence Gln-Ile-Val-Tyr-Lys-Pro (QIVYKP in Fig.2) was used to design the mixture of oligonucleotides that served as a hybridization probe to isolate a cDNA clone.

The invention includes assaying a sample from a patient for PHF core protein, a PHF core fraction or for an antibody to either. Thus, the assay (which may be used to diagnose Alzheimer's disease) may be conducted using PHF core protein or a precursor (or fraction) thereof as antigen to detect human antibody. The sample may be CSF or, especially in the case of an assay for an antibody, blood plasma or serum. It is envisaged that PHF core fractions, for example, might also be detectable in blood serum or plasma.

It will be understood that the invention is described above by way of Example only and modifications of detail may be made with the scope of the invention.

CLAIMS

1. A paired helical filament core protein substantially free of other proteinaceous material.

2. A paired helical filament core protein substantially free of the immunodominant protein layer.

3. A precursor or fraction of the paired helical filament core protein, substantially free of other proteinaceous material.

4. A separated paired helical filament core protein fraction.

5. A process for preparing a pronase-resistant paired helical filament residue, comprising:
   (i) isolating paired helical filaments from Alzheimer neurofibrillary tangles,
   (ii) digesting the isolated paired helical filaments with pronase, and
   (iii) purifying the insoluble pronase-resistant residue thereby obtained.

6. A process as claimed in claim 5, wherein step (i) comprises:
   (a) homogenising tissue containing neurofibrillary tangles, and
   (b) subjecting the filtered homogenate to a series of gradient centrifugation steps, whereby a fraction enriched in tangles and tangle fragments is obtained.

7. A process as claimed in claim 5 or claim 6, wherein step (ii) further comprises digesting the isolated paired helical filaments with nuclease.

8. A process as claimed in any one of claims 5 to 7, wherein step (iii) comprises subjecting the medium containing the digested filaments to density gradient centrifugation using a linear sucrose gradient poured between densities of 1.05 and 1.18 and collecting the fraction at the base of the gradient enriched in pronase-resistant residue.

9. A method as claimed in any one of claims 5 to 8, which further comprises subjecting the pronase-resistant residue to additional purification to prepare a separated core fraction.

10. A pronase-resistant paired helical filament residue prepared using a process as claimed in any one of claims 5 to 8.

11. A core fraction prepared using a process as claimed in claim 9.

12. An antibody to a core protein as claimed in claim 1 or claim 2 or to a residue as claimed in claim 10.

13. An antibody as claimed in claim 12 which is a monoclonal antibody.

14. The use of a core protein as claimed in claim 1 or claim 2 or a residue as claimed in claim 10 to prepare an antibody thereto.

15. An immunoassay involving an antibody as claimed in claim 12 or claim 13 for the presence in cerebrospinal fluid of (a) paired helical filament core protein or (b) a residue as claimed in claim 10, the antibody optionally being labelled.

16. A method for the diagnosis of Alzheimer's disease, comprising assaying a sample of cerebrospinal fluid for the presence of a paired helical filament core protein or a residue as claimed in claim 8.

17. A process for removing the "fuzzy" region of paired helical filaments (PHFs), comprising:

isolating paired helical filaments from Alzheimer neurofibrillary tangles by forming a preparation enriched therein,

homogenizing the enriched preparation in buffer,

digesting the homogenate with micrococcal nuclease and with pronase,

stopping the digestion,

adjusting the density of the digested mixture to 1.18,

subjecting the mixture to density gradient centrifugation at a density gradient of from 1.18 to 1.05 and over a cushion of CsCl solution, and

collecting the pronase-treated PHF fraction at the CsCl interface.

18. A process as claimed in claim 17, which further comprises re-centrifuging the pronase-treated PHF fraction to further purify it.

19. A process as claimed in claim 17 or claim 18, which further comprises:

sonicating the pronase-treated PHF fraction at pH 5.5,

centrifuging the sonicate and collecting the pellet,

sonicating the pellet in the presence of formic acid,

centrifuging the resultant mixture and collecting the supernatant,

lyophilising the supernatant,

sonicating the lyophilisate in an aqueous medium at pH 5.5,

centrifuging the sonicate and collecting the supernatant.

20. A process as claimed in claim 19, which further comprises subjecting the final supernatant material to an additional separation procedure.

21. A process as claimed in claim 17 or claim 18, which further comprises preparation of a PHF core fraction.

22. A material having immunological and electron microscopic structural characteristics corresponding to those of a pronase-treated PHF-fraction prepared by a method as claimed in claim 17 or claim 18.

23. A material having immunological and, if appropriate, electron microscopic structural characteristics of a PHF core fraction prepared by a method as claimed in any one of claims 19 to 21.

24. An antibody to a pronase-treated PHF fraction prepared or preparable by a method as claimed in claim 17 or claim 18 or to a material as claimed in claim 22.

25. A monoclonal antibody to (a) a PHF core fraction prepared or preparable by a method as claimed in any one of claims 19 to 21, (b) to a material as claimed in claim 23, (c) a precursor or fraction as claimed in claim 3 or (d) a fraction as claimed in claim 4 or claim 11.

26. The use to prepare an antibody thereto of a PHF-fraction prepared or preparable by a method as claimed in any of claims 17 to 21 or a material as claimed in claim 22 or claim 23.

27. An immunoassay involving an antibody as claimed in any one of claims 12, 13, 24 or 25.

28. A method for the diagnosis of Alzheimer's disease, comprising assaying a sample from a patient for the presence of a precursor or fraction as claimed in claim 3 or claim 4 or comprising performing an immunoassay as claimed in claim 27 on a sample from a patient.

29. A nucleotide probe to a nucleotide sequence encoding a core protein or a precursor or fraction thereof as claimed in any one of claims 1 to 4, to a material as claimed in claim 22 or 23, or to a pronase-treated PHF fraction preparable by a method as claimed in any one of claims 17 to 21.

5' -37 CCGCCTCTGTCGACTATCAGGTGAACTTTGAACCAGG

1 Met Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Asp His Ala Gly Thr Tyr Gly Leu Gly Asp Arg Lys Asp Gln Gly Gly Tyr Thr
1 ATG GCT GAG CCC CGC CAG GAG TTC GAA GTG ATG GAA GAT CAC GCT GGG ACG TAC GGG TTG GGG GAC AGG AAA GAT CAG GGG GGC TAC ACC

31 Met His Gln Asp Gln Glu Gly Asp Thr Asp Ala Gly Leu Lys Ala Glu Glu Ala Gly Ile Gly Asp Thr Pro Ser Leu Glu Asp Glu Ala
91 ATG CAC CAA GAC CAA GAG GGT GAC ACG GAC GCT GGC CTG AAA GCT GAA GAA GCA GGC ATT GGA GAC ACC CCC AGC CTG GAA GAC GAA GCT

61 Ala Gly His Val Thr Gln Ala Arg Met Val Ser Lys Ser Lys Asp Gly Thr Gly Ser Asp Asp Lys Lys Ala Lys Gly Ala Asp Gly Lys
181 GCT GGT CAC GTG ACC CAA GCT CGC ATG GTC AGT AAA AGC AAA GAC GGG ACT GGA AGC GAT GAC AAA AAA GCC AAG GGG GCT GAT GGT AAA

91 Thr Lys Ile Ala Thr Pro Arg Gly Ala Ala Pro Pro Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg Ile Pro Ala Lys Thr Pro Pro Ala
271 ACG AAG ATC GCC ACA CCG CGG GGA GCA GCC CCT CCA GGC CAG AAG GGC CAG GCC AAC GCC ACC AGG ATT CCA GCA AAA ACC CCG CCC GCT

121 Pro Lys Thr Pro Pro Ser Ser Gly Glu Pro Pro Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly Ser Pro Gly Thr Pro Gly Ser
361 CCA AAG ACA CCA CCC AGC TCT GGT GAA CCT CCA AAA TCA GGG GAT CGC AGC GGC TAC AGC AGC CCC GGC TCC CCA GGC ACT CCC GGC AGC

151 Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu Pro Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser Ser
451 CGC TCC CGC ACC CCG TCC CTT CCA ACC CCA CCC ACC CGG GAG CCC AAG AAG GTG GCA GTG GTC CGT ACT CCA CCC AAG TCG CCG TCT TCC

181 Ala Lys Ser Arg Leu Gln Thr Ala Pro Val Pro Met Pro Asp Leu Lys Asn Val Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu Lys His
541 GCC AAG AGC CGC CTG CAG ACA GCC CCC GTG CCC ATG CCA GAC CTG AAG AAT GTC AAG TCC AAG ATC GGC TCC ACT GAG AAC CTG AAG CAC

211 Gln Pro Gly Gly Gly Lys Val Gln Ile Val Tyr Lys Pro Val Asp Leu Ser Lys Val Thr Ser Lys Cys Gly Ser Leu Gly Asn Ile His
631 CAG CCG GGA GGC GGG AAG GTG CAA ATA GTC TAC AAA CCA GTT GAC CTG AGC AAG GTG ACC TCC AAG TGT GGC TCA TTA GGC AAC ATC CAT

241 His Lys Pro Gly Gly Gly Gln Val Glu Val Lys Ser Glu Lys Leu Asp Phe Lys Asp Arg Val Gln Ser Lys Ile Gly Ser Leu Asp Asn
721 CAT AAA CCA GGA GGT GGC CAG GTG GAA GTA AAA TCT GAG AAG CTT GAC TTC AAG GAC AGA GTC CAG TCG AAG ATT GGG TCC CTG GAC AAT

271 Ile Thr His Val Pro Gly Gly Gly Asn Lys Lys Ile Glu Thr His Lys Leu Thr Phe Arg Glu Asn Ala Lys Ala Lys Thr Asp His Gly
811 ATC ACC CAC GTC CCT GGC GGA GGA AAT AAA AAG ATT GAA ACC CAC AAG CTG ACC TTC CGC GAG AAC GCC AAA GCC AAG ACA GAC CAC GGG

301 Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly Ser Ile Asp Met
901 GCG GAG ATC GTG TAC AAG TCG CCA GTG GTG TCT GGG GAC ACG TCT CCA CGG CAT CTC AGC AAT GTC TCC TCC ACC GGC AGC ATC GAC ATG

331 Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Val Ser Ala Ser Leu Ala Lys Gln Gly Leu ***
991 GTA GAC TCG CCC CAG CTC GCC ACG CTA GCT GAC GAG GTG TCT GCC TCC CTG GCC AAG CAG GGT TTG TGA TCAGCCCCTGG 3'

FIG. 1

SUBSTITUTE SHEET

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        10        20        30        40        50        60        70        80
IKXVIXXGKVQIVYKPVDLSKVTSKSGSLGNIHHKPGGGQVEVKSEKLDFKDRVQSKIGDLGNIGGVPGGGNKKIET---TFRENAKAKT
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Fig 2.

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 88/00867

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC4: G 01 N 33/53, /68, C 12 P 21/00, C 07 K 15/00, C 12 Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC4	G 01 N; C 12 P; C 07 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X Y	Science, Vol. 235, 1987 Mori H. et al.: "Ubiquitin Is a Component of Paired Helical Filaments in Alzheimer's Disease ", see pages 1641-1644, in particular p. 1643, left col. lines 15-27 --	1,3,4 2,5,6,8-16, 27,28
X	Dialog Information Services, File 154:Medline 83-89/Jan, accession no. 06061696, Gorevic P D et al.: "Isolation and partial characterization of neurofibrillary tangles and amyloid plaque core in Alzheimer's disease: immunohistological studies. ", & J Neuropathol Exp Neurol, Nov 1986, 45 (6) p. 647-64 --	1-5
Y	The Journal of Cell Biology, Vol. 100, 1985 Wischik C M et al.: "Subunit Structure of Paired Helical Filaments in Alzheimer's Disease. ", see page 1905 - page 1912, in particular p. 1906, left col. --	1-6,8-16

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11th January 1989	30 JAN 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

International Application No. PCT/GB 88/00867

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Acta Neuropathologica, Vol. 74, 1987 Bancher C et al.: "Neurofibrillary tangles in Alzheimer's disease and progressive supranuclear palsy: antigenic similarities and differences. ", see page 39 - page 46 --	1-5,12-16
Y	Dialog Information Services, File 154:Medline 83-89/Jan, accession no. 06111614, Ksiezak-Reding H et al.: "Two monoclonal antibodies recognize Alzheimer's neurofibrillry tangles, neurofilament, and microtubule-associated proteins. ", & J.Neurochem,Feb 1987, 48 (2) p. 455-62. --	12-14
A	British Medical Bulletin, Vol. 42, No. 1, 1986 C M Wischik et al.: "Subunit Structure of the Alzheimer Tangle. ", see page 51 - page 56, in particular p. 53-55 --------	1-5,12-16